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(54) Title: EXPRESSION OF HETEROLOGOUS PROTEINS BY TRANSGENIC LACTATING MAMMALS

(57) Abstract

Mammals capable of expressing recombinant proteins by lactation are produced by microinjection of recombinant DNAs containing novel expression systems into fertilized ova.

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TITLE

Expression of Heterologous Proteins by Transgenic Lactating Mammals

BACKGROUND OF THE INVENTION

The present invention relates generally to transgenic animals, and particularly to methods of producing recombinant proteins as components of the milk of lactating transgenic mammals.

Currently, recombinant proteins are produced predominantly by inserting selected genes (cDNAs) into phage, plasmid, or viral expression vectors, which direct production of the desired protein products in bacteria, yeast, or mammalian cells grown in culture. Typical expression systems provide product concentrations ranging from 10 to 100 mg recombinant protein per liter fermentation or culture fluid. However, additional product losses are encountered in downstream purification and processing steps, during which the recombinant protein is isolated from fermentation or culture media. For these reasons, protein products which are used in extremely large quantities have not been produced by recombinant DNA technologies; the yields available from known expression systems fall short of providing commercially feasible processes.

Certain proteins are, however, naturally produced at high expression levels. For example, lactating mammals can accumulate 5-20 grams per liter of particular proteins in their milk. The present invention provides systems and methods for harnessing the high level protein expression of lactation to produce recombinant proteins.

SUMMARY OF THE INVENTION

The present invention provides a recombinant expression system comprising a lactogen-inducible mammalian genomic regulatory region and a structural region encoding a heterologous protein. In a related aspect, the invention concerns transgenic mammals having mammary secretory cells incorporating a recombinant expression system comprising a mammalian lactogen-inducible regulatory region and a structural region encoding a heterologous protein. Such a regulatory region can comprise, for example, nucleotide sequences which are substantially homologous to sequences located 5′ with respect to an intact native milk protein gene. In a process aspect, the invention provides a method of producing a recombinant protein, comprising obtaining milk from a lactating transgenic mammal having the foregoing characteristics, and isolating recombinant protein from the milk.

DETAILS OF THE INVENTION

Advances in technologies for embryo micromanipulation now permit transfer of heterologous DNA into fertilized mammalian ova. Microinjection into the male pronucleus of a fertilized egg typically results in chromosomal integration of the injected DNA in the form of tandem multiple copies, or concatemers, at single, random sites. Species differences in the manipulability of mammalian eggs affect the success of obtaining viable embryos for reimplantation following microinjection or fertilization in vitro. However, both murine and bovine embryos can be fertilized in vitro, manipulated, and grown in culture to the blastocyst stage prior to implantation. Thus, a number of workers have reported successful experiments involving transgenic mice [for a review see Palmiter et al., Cell 41:343 (1985)]. Most genes that have been microinjected into murine embryos are expressed; levels of expression vary between individuals.

The present invention relies upon microinjection and embryo transfer technologies to generate transgenic individuals having a novel genomic protein expression system. As noted above, the expression system comprises selected regulatory elements derived from intact milk protein genes, fused to a structural gene encoding a heterologous protein which is to be produced in large quantity. The objective is to attain a transgenic individual having mammary secretory cells capable of producing the heterologous protein in response to lactogenic hormones or stimuli. In short, recombinant protein is expressed in and recovered from the milk of the transgenic mammal.

As used throughout the specification, "recombinant expression system" refers to an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers, and (2) a structural genetic element or elements, comprising a coding sequence(s) which is transcribed into mRNA and translated to mature protein. Thus, "regulatory region" refers to the segments of the expression system, or alternatively, to segments of native genes, which comprise such regulatory elements. The regulatory region of a particular system can include sequences located both 5' and 3' with respect to a particular structural sequence, as well as intervening sequences, or "introns", which are loci interrupting a structural sequence. The terms "structural region" or "structural sequence" refer to DNA segment(s) encoding a particular protein, and can include a nucleotide sequence encoding a signal or leader polypeptide, which may or may not be heterologous with respect to the remainder of the coded protein. Such a signal or leader polypeptide may be required in secretion of expressed protein from particular cells. "Heterologous protein" refers to any protein not naturally associated with the regulatory elements employed in the recombinant expression system. The term

"substantially homologous", which can refer both to nucleic acid and amino acid sequences, means that a particular sequence is identical to a reference sequence, or varies from the reference sequence by one or more substitutions, deletions or additions, the net effect of which do not result in an adverse functional dissimilarity between biological activity of reference and subject sequences. The term "milk protein" is a generic term comprehending native proteins, produced and secreted by mammary cells in response to lactogenic stimuli. Examples of milk proteins are α-lactalbumin, α-casein, β-casein, and γ-casein.

1. Overview of System Assembly

The first step in constructing the expression system of the present invention involves isolating a gene coding for a preselected milk protein, for example, the bovine α-lactalbumin gene, from a library of appropriate genomic DNA. To this end, a DNA probe is synthesized, comprising a nucleotide sequence corresponding to known sequences of a corresponding gene, e.g., rat α-lactalbumin. A group of clones selected from the library are then analyzed by restriction mapping and nucleotide sequencing. Fragments likely to contain regulatory or structural sequences of the gene of choice are tentatively identified by comparision to the known sequences of homologous or related genes.

Simultaneously, a cDNA library obtained from mammary tissues of lactating cows can be screened with the oligonucleotide probes to isolate a cDNA correponding to the structural sequence of the milk protein, less any introns.

After isolating clones from the genomic and cDNA libraries presumed to contain the regulatory and structural sequences of interest, a series of studies are undertaken to determine which regions are required to regulate expression of the milk protein gene. The essential regions are identified by assembling plasmids which contain the putative

regulatory sequences joined to a suitable indicator gene, as well as sequences enabling stable transformation of mammalian host cells. Mammary cells capable of induction by lactogenic hormones are then exposed to the recombinant constructs under conditions which promote uptake and incorporation of foreign DNAs. Expression of the indicator gene under lactogenic inducing conditions is then monitored by an assay specific for the indicator gene product.

Using this approach, the essential regulatory regions can be identified. If regulatory sequences are not apparent by homology to known regulatory regions of other lactogenic hormone-controlled genes, then further sequences from the gene, including 5' and 3' flanking sequences and intron sequences, can be systematically assayed for their ability to confer lactogenic hormone inducibility upon the indicator gene when linked to it.

As a direct test of the essentiality of intron sequences present in the intact gene, a construct consisting of the 5' and 3' non-coding sequences joined to a cDNA corresponding to the protein product can be introduced to host cells, and expression of the milk protein gene measured using a specific antibody.

Once the essential lactogen-inducible regulatory elements have been identified, new constructions comprising these elements in conjunction with heterologous structural sequences can be assembled and tested for expression in cultures of transformed host cells.

After testing for expression in cell cultures, expression systems comprising either the milk protein gene with introns removed or heterologous structural sequences are introduced to fertilized ova of a chosen species by microinjection in vitro, and the resulting microinjected zygotes implanted in the reproductive tract of a pseudopregnant female. Following gestation and delivery, the transplanted offspring can be genetically screened for genomic incorporation of the expression system, using

polynucleotide probes. Finally, expression of the milk protein gene or heterologous protein in the milk of mature transgenic animals is confirmed by appropriate assay.

The following disclosure of methods for assembling and using the expression systems of the present invention describes isolation and cloning of lactogen-inducible regulatory elements associated with the bovine α-lactalbumin gene. However, it is to be understood that this disclosure is merely exemplary, and not limiting with respect to the actual scope of the invention. Thus, a parallel approach to that described below could be adopted in assembling expression systems comprising other lactogen-inducible regulatory elements, for example, genes of the casein family.

The numerous published references cited herein are expressly incorporated by reference. Citation of a reference also provides implicit recognition that the techniques disclosed therein are within the skill of the art, thereby constituting nonessential subject matter for purposes of the present disclosure. Proteolytic enzymes, restriction endonucleases, RNAse H, E. coli DNA ligase, T4 polynucleotide kinase, DNA polymerase I, reverse transcriptase, expression plasmids such as pBR322, bacterial host strains, phage cloning vectors, kits for nick-translation of DNA, and other reagents and materials employed in the procedures described below are commercially available from numerous vendors.

2. Isolation of Bovine α-lactalbumin Gene

A bovine genomic library is constructed in λ phage and screened with oligonucleotide probes by conventional techniques, for example, those disclosed by Maniatis et al., et al., Molecular Cloning, A Laboratory Manual, (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982; hereinafter "Maniatis").

A set of 32P-labeled oligonucleotide probes representative of the signal sequence region, 5′ non-coding region, and the N-terminus of the known sequence of the rat α-lactalbumin gene are prepared by standard oligonucleotide synthesis techniques. Suitable techniques for oligonucleotide triester synthesis are described by Sood et al., Nucleic Acids Res. 4:2557 (1977) and Hirose et al., Tet. Lett. 28:2449 (1978); following synthesis, probes are labelled using 32P-ATP and T4 polynucleotide kinase substantially as described by Maniatis. The complete nucleotide sequence of rat α-lactalbumin gene is disclosed by Qasba et al., Nature 308:377 (1984). Preferably, the probes are at least 60-mers to ensure relatively efficient trans-species hydridization to DNA fragments comprising the bovine α-lactalbumin gene.

Restriction mapping and DNA sequencing are employed to identify the bovine genomic fragments encoding the entire bovine α-lactalbumin gene, including at least 1000 base pairs 5′ to the start of the transcription initiation codon, the 5′ transcribed but non-coding sequences, the coding or structural region, and the 3′ non-coding region. These regions are identified by homology with the known sequence of the rat α-lactalbumin gene, and also by reference to the sequence of a bovine α-lactalbumin cDNA, if available. Conventional methods for DNA chain-termination sequence determination are described in the Amersham handbook M13 Cloning and Sequencing (Blenheim Crescent, London 1983); and by Messing, Recombinant DNA Tech. Bull. 2:43 (1979); and Norrander et al., Gene 26:101 (1983).

A bovine mammary cDNA library can be contructed from bovine mammary parenchymal tissue obtained from the mammary organs of lactating cows at slaughter. The isolated tissue is mechanically comminuted and treated with collagenase, hyaluronidase, or other suitable dispersing aids to generate a cell suspension. Polyadenylated

messenger RNA is then isolated from the parenchymal cell suspensions by methods substantially similar to the guanidium thiocyanate and guanidine hydrochloride methods disclosed by Maniatis and by Strohman et al., Cell 10:265-273 (1977).

Double-stranded cDNA is then synthesized from the polyadenylated RNA fractions by standard techniques (Maniatis), and a cDNA library created in plasmid or phage, for example, pBR322. Oligonucleotide probes corresponding to structural sequences of the rat α-lactalbumin gene can then be used to screen the library for clones bearing bovine α-lactalbumin cDNA.

3. Identification of Lactogen-Inducible Regulatory Elements

In order to identify the regulatory elements required to provide lactogen-inducible expression of particular structural sequences, plasmids are constructed which contain sequences located 5′ with respect to the expected transcription initiation site, as well as part of the 5′ non-coding region, fused to a selected indicator gene.

The bacterial chloramphenicol 3-O-acetyltransferase gene, or CAT, is particularly useful for this purpose. The enyzyme encoded by this gene can be sensitively and accurately quantified by the method of Gorman et al., Mol. Cell. Biol. 2:1044 (1982), which is based upon conversion of chloramphenicol to an acetylated form. Since the enzyme has no counterpart in eukaryotic cells, the amount of enzyme produced after transfection of an appropriate construct into eukaryotic host cells reflects the level of expression provided by a particular promoter or other regulatory element. A plasmid containing the CAT gene, pSV2-CAT is described by Howard et al., Proc. Nat. Acad. Sci. USA 79:6777 (1982), and is available from the ATCC under accession number 37155. Alternative indicator elements can be employed. For example, if specific antibody is available

[see Hopp et al., Mol. Immun. 19:1453 (1982)], expression of the intact bovine α-lactalbumin gene could be assayed in cell extracts.

The test construct preferably contains an additional sequence enabling RNA splicing and polyadenylation following introduction to recipient mammalian cells. A preferred sequence for this purpose is the simian virus 40 (SV40) small-T antigen splice donor and acceptor sites (SV40 coordinates 4,035-4656) and the SV40 polyadenylation site (SV40 coordinates 2,469-2,706). These elements can be obtained as a BglII-BamH1 fragment from plasmid pSV2-dhfr (ATCC 37145), described by Subramani et al., Molec. Cell. Biol. 1:854 (1981).

The plasmid thus created is then introduced to recipient cells likely to respond to lactogenic hormones. For example, primary bovine mammary cell cultures can be employed, or cultures of mouse mammary cells such as the cell line COMMA-1D described by Danielson et al., Proc. Natl. Acad. Sci. USA 81:3756 (1984). The COMMA-1D cells have been shown to induce synthesis of murine caseins in response to lactogenic hormones. As positive, non-inducible controls, plasmids containing the SV40 elements and CAT indicator, but lacking inserted regulatory elements, are employed in parallel transfection experiments.

Effective techniques for transfection of mammalian cells in culture include those disclosed by McCutchan et al., J. Natl. Cancer Inst. 41:351 (1968); Graham et al., Virology 52:46 (1973); Frost et al., Virology 103:369 (1978); and Luthman et al., Nucleic Acids Res. 11:1295 (1983).

Transfected cells are collected by scraping and resuspended in induction medium, which consists of growth medium containing prolactin (5 <g/ml), aldosterone (5 <g/ml), and hydrocortisone (1 <g/ml). The cultures are incubated at 37°C for four days in induction medium (changed daily) and then extracts of the treated cells are tested for

chloramphenicol transferase activity by the assay previously referenced. This procedure is repeated with various combinations of the putative regulatory elements selected by hybridization, as described above, until each element required for lactogen-induced expression of the CAT gene has been identified.

4. Assembly of Expression System for Interleukin-2

After trial assemblies of the α-lactalbumin regulatory elements and heterologous indicator protein or α-lactalbumin cDNA are shown to provide lactogen-inducible expression in cell culture, second-generation assemblies of the α-lactalbumin regulatory region and other structural sequences can be tested. For example, a structural sequence coding for human interleukin-2, a regulatory hormone of the immune system can be obtained by preparation of a cloned cDNA as described by Taniguchi et al., Nature 302:305 (1983). A plasmid is then constructed, containing the interleukin-2 sequence and a signal sequence derived from the native interleukin-2 gene or the bovine α-lactalbumin gene, in conjunction with the α-lactalbumin regulatory elements. The plasmid is amplified in cultures of appropriate recipient cells, and a preparation of purified plasmid DNA cleaved with an appropriate restriction enzyme to provide a linear fragment containing the α-lactalbumin regulatory region, α-lactalbumin (or IL-2) signal peptide, and interleukin-2 structural regions. This fragment is isolated by electrophoresis on an agarose gel and reserved for injection into fertilized mouse ova.

Similarly, a linear fragment comprising the bovine α-lactalbumin cDNA and α-lactalbumin regulatory region can be prepared for parallel experiments to determine expression of an intron-free bovine α-lactalbumin gene by transgenic mice.

5. Microinjection of Mouse Zygotes

Six week-old female mice are induced to superovulate by injection of 5 international units of pregnant mares' serum, followed 48 hours later by 2.5 international units human chorionic gonadotropin, and placed immediately with males for mating. Approximately 14 hours following mating, those females exhibiting vaginal plugs are sacrificed and their oviducts removed and placed in Krebs-Ringer bicarbonate buffered medium, containing bovine serum albumen and hyaluronidase at 1 mg/ml. Oviducts are opened with forceps and fertilized eggs and remaining follicle cells are expressed into a culture dish. After 1-2 minutes, eggs are removed and washed with culture medium previously equilibrated with 5% CO2 in air at 37°C. Eggs containing pronuclei are identified under a dissecting microscope and placed in lots of 20 in a microdrop of equilibrated medium, which is then placed in a 100 mm culture dish and covered with mineral oil. Eggs are stored in the incubator in this manner until microinjected.

Microneedles having a tip diameter of about 1-2 <m are pulled from thin-walled glass tubing using a pipette puller. Holding pipettes (for holding eggs) having a tip diameter of 60-70 <m are similarly pulled from capillary tubing, and the ends fire polished using a microforge. The tips of the microneedles are allowed to fill with a suspension of plasmid DNA by capillary action. The holding pipettes and microneedle barrels are filled with an inert fluorocarbon (Fluorinert, 3M), and each microneedle and holding pipette is then secured to polyethylene tubing of appropriate diameter, which is in turn fitted to 1 mL Hamilton syringes secured in micromanipulators. Both microneedle and holding pipette apparatus are secured to the stage of a light microscope having a 1200x objective.

The culture dish containing the suspended zygotes is secured to the microscope stage in proximity to the microinjection apparatus, and a microneedle containing

plasmid solution is moved close to the drop containing the zygotes. A zygote is then positioned on the holding pipette such that the male pronucleus is in focus, and the microneedle slowly inserted into the pronucleus. Sufficient plasmid suspension, (about 2 pl) is injected to approximately double the size of the pronucleus, and then the microneedle is slowly withdrawn. This procedure is repeated with the remaining fertilized eggs.

After an additional hour of incubation, surviving eggs are transferred to the oviducts of plugged pseudopregnant female mice as follows. Each foster female is anethetized with 6 mg/100g sodium pentobarbital, and ovaries are located through a dorsal incision. The ovarian bursa is dissected from the supporting tissues with forceps, and the ostium of the oviduct visualised under the dissecting microscope. A pipette containing 10-20 microinjected embryos is inserted into the oviduct, and the wound closed. Approximately 20 days later, mice are examined for delivery of live offspring.

After weaning, tail tips are taken from offspring and high molecular weight DNA isolated by the method of Blin et al., Nucleic Acids Res. 3:2302 (1976). The isolated DNA's are then screened for the presence of heterologous DNA by the dot-hybridization method of Kafatos et al., Nucleic Acids Res. 7:1541 (1979), using nick-translated fragments of the cloned plasmid DNA used in the microinjection experiments. On this basis, transgenic animals are identified and isolated. At about six weeks of age, female transgenic mice are injected with a lactogenic inducing mixture of prolactin, aldosterone and hydrocortisone. Milk is expressed from the mammary glands and tested by appropriate assay for the presence of bovine α-lactalbumin or human interleukin-2.

6. Expression in other species

The foregoing techniques are employed to tranform species of particular interest, such as cows and goats, using substantially similar techniques. However, microinjection of DNA containing the expression system of the invention into the male pronuclei of fertilized ova is critical to success. For this reason, visualization of the male pronucleus is necessary. In goat ova, male pronuclei can be visualized using interference contrast microscopy. The male pronuclei of bovine zygotes can be visualized following centrifugation for 3 min. at 15,000 x g. Further details regarding the mechanics of microinjection and embryo transfer in livestock are provided by Hammer et al., Nature 315:680 (1985); Wall et al., Biol. Reprod. 32:645 (1985); and Brinster et al., Cell 27:223 (1981).

CLAIMS

What is claimed is:

1. A recombinant expression system comprising a lactogen-inducible mammalian genomic regulatory region and a structural region encoding a heterologous protein.

2. A recombinant expression system according to Claim 1, wherein the regulatory region comprises nucleotide sequences which are substantially homologous to sequences derived from an intact milk protein gene.

3. A recombinant expression system according to Claim 2, wherein the milk protein is α-lactalbumin.

4. A recombinant expression system according to Claim 3, wherein the α-lactalbumin gene is a bovine α-lactalbumin gene.

5. A transgenic mammal having mammary secretory cells incorporating á recombinant expression system comprising a mammalian lactogen-inducible regulatory region and a structural region encoding a heterologous protein.

6. A transgenic mammal according to Claim 5, wherein the regulatory region comprises nucleotide sequences which are substantially homologous to sequences derived from an intact milk protein gene.

7. A transgenic mammal according to Claim 6, wherein the milk protein is α-lactalbumin.

8. A transgenic mouse according to Claim 7.

9. A transgenic cow according to Claim 8.

10. A transgenic mouse according to Claim 9, wherein the lactogen-inducible construct comprises non-coding regulatory sequences which are substantially homologous to the non-coding regulatory sequences of a bovine α-lactalbumin gene.

11. A transgenic cow according to Claim 9, wherein the lactogen-inducible construct comprises non-coding regulatory sequences which are substantially homologous to the non-coding regulatory sequences of a bovine α-lactalbumin gene.

12. A method of producing a recombinant protein, comprising obtaining milk from a lactating transgenic mammal according to Claim 5, and isolating recombinant protein from the milk.

13. A method of producing a recombinant protein, comprising obtaining milk from a lactating transgenic cow according to Claim 11, and isolating recombinant protein from the milk.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/02069

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12P 21/00, 21/02; C12N 15/00, 1/00
US CL : 435/68,70,172.3,320; 800/1; 935/34,111

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/68,70,172.3,320; 800/1; 935/34,111

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

COMPUTER SEARCH CAS, APS: LACTOGEN, TISSUE SPECIFIC EXPRESSION, TRANGENIC

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	Nucleic Acids Research Vol. 14 issued 25 February 1986, (Oxford England), (YU-LEE ET AL) "Evolution of the casein multigene family: Conserved sequences in the 5' flanking and exon regions" pages 1883-1902.	1-2 3-13
X,P Y,P	Biochemical Journal, Vol. 242, issued January 1987, (London England), (HALL ET AL), "Organization and sequence of the human α-lactalbumin gene", pages 735-742.	1-3 4-13
X Y	Nature, Vol. 308 issued 22 March 1984, (London England), (QASBA ET AL), "Similarity of the nucleotide sequences of rat α-lactalbumin and chicken lysozyme genes"," pages 377-380.	1-3 4-13

* *Special categories of cited documents:* [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
28 October 1987	30 NOV 1987.
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	Alvin E. Tanenholtz

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	Cell, Vol. 38 issued October 1984 (Cambridge Massachusetts, USA), (SWIFT ET AL), "Tissue-Specific Expression of the Rat Pancreatic Elastase 1 Gene in Transgenic Mice," pages 639-646.	1-13
Y	Proc. Natl. Acad. Sci. USA, Vol. 83, April 1986, (Washington, D.C.), (BUCCHINI ET AL), "Pancreatic expression of human insulin gene in transgenic mice," pages 2511-2515.	1-13
Y	Cell, Vol. 41 issued June 1985 (Cambridge Massachusetts), (PALMITER ET AL), "Transgenic Mice," pages 343-345.	1-13